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14. ABSTRACT
Endothelial cells from different vascular beds, including tumor vasculature, have been shown to have different behavior and different gene expression. In tumors, abundant fibrin in the extracellular matrix dictates that fibrinolytic capability is required for new vessel formation. We have developed an in vitro breast cancer angiogenesis assay using mammary vessels from mice in a 3-dimensional fibrin matrix. Confocal microscopy of these assays is used to quantitate the number of endothelial cells invading the matrix as well as the length of tubular structures formed by the cells. This assay can be used to investigate fibrinolytic molecules important in vascularization of breast tumors.

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Angiogenesis, endothelial, plasminogen, fibrinolysis

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Introduction

Tumor-associated blood vessels are abnormally leaky and allow the extravasation of fibrinogen and other components of the coagulation system. Exposure of these procoagulants to extravascular matrix components and procoagulant tumor products activates the coagulation cascade and deposits large amounts of fibrin. Therefore, in tumors, angiogenic endothelial proteolytic capability would have to include fibrinolysis. We have isolated pure populations of tumor endothelial cells from xenograft breast tumors and from mammary fat pads of nude mice. RNA obtained from tumor- and mammary fat pad-associated endothelial cells was used to synthesize cDNA and cDNA libraries, which were used in differential cloning techniques to compare gene expression in tumor-associated endothelial cells with that of mammary fat pad endothelial cells. Preliminary results showed upregulation of three genes associated with fibrinolytic capability, tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and membrane type matrix metalloproteinase-1 (MT1-MMP), in tumor-associated endothelial cells but not in mammary fat pad endothelial cells. In these preliminary studies, urokinase plasminogen activator (uPA), its receptor (uPAR), and the tPA receptor, annexin II, were each constitutively expressed. We hypothesized that tumor endothelial cells acquire additional fibrinolytic capability during the process of angiogenesis, and this activity is important for the morphologic transformations accompanying the angiogenic process.

We started our studies because of the data that tumors are rich in fibrin and because of the link of urokinase plasminogen activator (uPA) expression to poor prognosis[1-5]. Because there was little data from tumor models or from in vitro assays in fibrin, we felt that our proposed investigations would shed light on the role of the fibrinolytic system in breast cancer angiogenesis. Tumors have substantial fibrin as part of their extracellular matrix and invading endothelial cells necessarily must invoke fibrinolytic activity to invade the fibrin to form new tubular structures. However, studies in "knock-out" mice have shown very little change in the vascular system for mice deficient in tissue plasminogen activator (tPa), uPA, urokinase plasminogen activator receptor (uPAR), or plasminogen activator inhibitor-1 (PAI-1)[6]. However, PAI-1 deficient mice were shown to vascularize tumors less efficiently, with a resultant decrease in tumor size when compared to similar tumors in wild-type mice[7]. Additionally, MT1-MMP deficient mice do have a vascular phenotype[8], and this enzyme has been shown to be an important fibrinolytic enzyme. In summary, although the literature implicates the fibrinolytic system as an indicator of poor prognosis, only MT1-MMP deficiency has been shown to produce a vascular phenotype, and only PAI-1 has been implicated as a factor in tumor-associated angiogenesis.

The literature also robustly supports the notion that endothelial cells from vessels in different organs and different types of vessels have specific patterns of gene expression[9]. We therefore felt that aortic ring assays previously done as *in vitro* models of angiogenesis may have been producing data that possibly did not apply to breast cancer endothelium since endothelial cells in these assays do not come from a venule in the breast. Our previous studies (above) showed that breast tumor-associated endothelial

cells have strongly upregulated messenger RNA (mRNA) for tPA and MT1-MMP, with some upregulation of uPA expression.

BODY

1. Confirm differential expression of tPA and MT1-MMP with GAPDH control.

The preliminary results showing upregulation of tPA and MT1-MMP that formed the basis of the proposal were done on plasmid cDNA libraries synthesized from the isolated tumor-associated and mammary fat pad endothelial cells. They were done with equal amounts of plasmid DNA with a number of cycles that could be expected to maintain logarithmic amplification. In working with these plasmid libraries since then, it is apparent that the mammary fat pad endothelial cell library is not as diverse as the tumor-associated endothelial cell libraries. This is most likely due to the extremely small numbers of endothelial cells isolated from the mammary fat pads that formed the basis of the library. In order to achieve a more accurate representation of the relative expression of genes of interest in the libraries, they were standardized to GAPDH expression. Under these conditions, the differential expression of tPA and MT1-MMP was confirmed but it appears that uPA is also upregulated in tumor-associated endothelial cells. uPAR may be somewhat upregulated (Figure 1) but the degree of upregulation is slight, and could be due to variability in the PCR reaction. Moreover, one cannot consider the plasmid endothelial cell libraries to be complete representative of gene expression in the cells from which they were derived because of losses that may have occurred during library construction. Nonetheless, these data provide evidence to support our hypothesis that fibrinolytic capability is upregulated in tumor-associated endothelial cells.

2. Adaptation of the aortic ring assay to mammary fat pad vessels.

Dr. Passaniti, collaborator on the project, has extensive experience with the aortic ring assay[10] using a fibrin matrix. Under his tutelage, we have adapted the assay to mammary vessels. Six to eight week old female nude mice are implanted with estrogen pellets (0.72 mg, 60 day release) to stimulate development of the mammary gland and to approximate the hormonal conditions of tumor growth. Two weeks after pellet implantation, mice are sacrificed and the mammary vessels of the inguinal mammary fat pad dissected, trimmed free of as much fat as possible, cut into small segments, and embedded in fibrin (Figure 2 & 3). If tumor cells are desired as angiogenic stimulus, they are embedded in a pellet of collagen prior to the assay, and placed in one corner of the chamber, while the mammary vessel fragment is in an opposite corner. Many cells leave the explant and invade the fibrin. Some cells form into tubes while others do not (Figure 4). We have been able to use DiI-labeled LDL uptake to further identify endothelial tubes (Figure 5B). This has been possible because of a unique fixation technique utilizing a combination of paraformaldehyde fixation with detergent and Dent's fixative, a dimethylsulfoxide/methanol protocol[11].

3. Fluorescence studies and confocal analysis of mammary vessel assays.

We have been able to identify endothelial tubular structures in the 3-dimensional fibrin matrix by using uptake of DiI-labeled acetylated low-density lipoproteins (DiI-acLDL). Acetylated LDL does not internalize as a result of binding to the LDL receptor but rather because of a scavenger receptor that is specific to endothelial cells[12]. We have been working to compare results in this system using mammary vessel explants or aortic ring explants. These studies will confirm our belief that mammary vessels may respond to angiogenic factors differently than aortic rings and validate the specificity of our assay.

We were able to obtain positive immunofluorescence for PECAM-1, a membrane protein expressed by endothelial cells at relatively high levels (Figure 5C). However, our ability to stain this protein in endothelial cells in fibrin gels has been inconsistent, in spite of our varying many of the fixation and staining conditions in a multitude of permutations and combinations. Moreover, when we attempt to stain a less abundantly expressed protein, the results are uniformly negative. As mentioned above, we are able to identify endothelial tubes in the mammary vessel assays by virtue of their ability to specifically take up DiI-labeled acetylated LDL. The DiI label imparts a red fluorescence to the endothelial cells (Figure 5B). This method of identifying endothelial tubes in the mammary vessel assays has the advantage that it can be used for *in vivo* staining during the culture period, before fixation. In this way, the progress of the endothelial cell outgrowths can be monitored in the culture and cultures harvested for fixation at opportune times. DiI-AcLDL labeled structures can be quantified by image analysis to measure degree of outgrowth. We have concluded that DiI-AcLDL labeling is a better way to identify endothelial cells in cultures than immunofluorescence for PECAM-1. Because the DiI-AcLDL labeling can be observed in live cultures (Figure 6), we can acquire images during assay culture and quantify DiI-AcLDL labeled structures at different time points for the same assay.

3A. Do growth factors alter DiI-labeled acLDL uptake?

In order to quantitate the number of sprouts in each chamber with DiI-labeled acLDL, we would want to know that BPE, FGF, or VEGF treatment did not affect uptake of DiI-labeled acLDL. To ascertain this, we used an immortalized microvascular cell line, HMEC-1. These cells were plated in 75-mm^2 tissue culture flasks, treated with BPE, VEGF, and/or FGF similarly to the explants, and exposed to AlexaFluor 488 - labeled acLDL (Molecular Probes). Cells were harvested and analyzed with flow cytometry. This analysis is depicted in Figure 7. It is seen that fluorescence from the Alexa Flour 488-acLDL in the presence or absence of treatments is not substantially different.

3B. Is ε-amino-n-caproic acid (ACA) necessary for fibrin matrix assays or aortic rings or mammary vessels?

Others[13] have added ACA, a plasminogen inhibitor, to fibrin matrix assays at low concentrations to prevent complete digestion of the fibrin due to activated plasminogen that is the result of tissue damage consequent to dissection of the explant. These investigators have performed analyses of fibrin digestion by use of blocking antibodies to particular plasminogen activators or inhibitors, so it does not seem that this agent interfered with the analyses. However, we were concerned that this agent might be a complicating factor for our assays and wished to see whether we could eliminate it. We performed a concentration-response of ACA starting at 300 μg/ml and going down to 10 μg/ml as well as leaving it out of one assay completely. We did not observe any difference between the chambers that contained various concentrations of ACA or the chambers that contained no ACA (not shown). Therefore, for our assays, it would seem that this agent can be omitted and it will be omitted in future experiments.

3C. Responses of mammary vessels and aortic rings growing in fibrin matrices to angiogenic factors. As outlined in the original proposal, we first studied the mammary vessel explants in endothelial cell media without additions or with various additives. Table 1 outlines the media used for these experiments. Each assay consisted of 4 chambers in a chambered coverslip. Mammary vessels were placed in fibrin matrices in assays 1-8 and aortic rings were similarly treated in assays 9-16. All 4 chambers were treated with DiI-AcLDL for 3 days prior to harvest. Assays were fixed with 4% paraformaldehyde/1.5% Triton-X 100 overnight at 4°C. After washing with PBS, Dent's fixative was applied overnight at 4°C, followed by Dent's fixative plus 6% hydrogen peroxide for 2 hours at room temperature with rocking. Chambers were then washed with 100% methanol twice and incubated at -80° overnight. After two washes with 50% methanol in PBS for 30 minutes at room temperature two similar washes with phosphate-buffered saline (PBS) plus 0.5% Triton-X 100, chambers were stained with Yo-Pro-1 (1:750 in PBS), which binds to nucleic acids and fluoresces green. Depending on the intensity of excitation, Yo-Pro-1 produces intense green nuclear staining and less intense cytoplasmic staining. Fluorescence microscopy was performed with the excitation laser for Yo-Pro-1 set to give nuclear, but not cytoplasmic staining.

Assay number	Growth Factor/conc.
1 & 9	No additives
2 & 10	BPE
3 & 11	VEGF, 50 ng/ml
4 & 12	FGF ng/ml
5 & 13	BPE + VEGF
6 & 14	BPE + FGF
7 & 15	VEGF + FGF
8 & 16	All

Table 1. Additives to fibrin matrices for mammary vessels or aortic rings. Chambers 1-8 were mammary vessels and chambers 9-16 were aortic rings. Basal media was 199E with 10% fetal bovine serum, 0.5 ng/ml EGF, and 3 μM hydrocortisone. BPE, bovine pituitary extract; VEGF, vascular endothelial growth factor; FGF, basic fibroblast growth factor (FGF-2).

Figure 8 shows representative low-power fields (4X objective with 2.5X zoom) of mammary vessel (MFP) and aortic ring (AR) explants growing in fibrin matrices with treatments as given in Table 1. The explant is the large red structure at the side, top or bottom of each picture. A square with "no sprouts" signifies that none of the four chambers of the chambered cover slip containing the vessel (in all cases, aortic ring) fragments exhibited sprouting from the explant. Interestingly, the mammary vessels exhibited endothelial sprouting even in media supplemented only with EGF,

hydrocortisone, and 10% fetal calf serum while the aortic rings did not. Although supplementation with bovine pituitary extract (BPE) enabled sprouting in both aortic rings and mammary vessels, addition of BPE to VEGF and basic FGF (FGF) supplementation seemed inhibitory in both vessel types. Although representative fields are shown, the differences produced by different treatments and the difference between mammary vessels and aortic rings are more pronounced than can be seen in Figure 8. For instance, in Figure 8 with the combination of FGF and VEGF (next to the last column of pictures), the field depicting aortic ring sprouts (bottom row) represents the only sprouting we saw in aortic rings with this treatment. However, the mammary vessels treated with the combination of FGF and VEGF exhibited sprouting in every chamber and at multiple locations from the same explant. We are in the process of devising an image analysis macro using IMAGEJ software (a free download from the NIH) to quantitate the number of sprouting cells. An example of this is shown in Figure 9. This should reveal quantitative differences between treatments. Additionally, using confocal microscopy, we have acquired high power (40X water-immersion objective) z series on each a number of fields from each chamber. These have been used to generate 3-dimensional projections that show tubular structures formed by the sprouting endothelial cells (Figure 10). These structures can be analyzed as to length and branch points by the ImageJ software. Over the few months, we will be analyzing the structures in the sprouts to develop a more quantitative picture of high the aortic rings and mammary fat pads differ.

4. Construction of sense and antisense retroviral vectors:

We proposed to abrogate MT1-MMP and tPA expression in mammary vessel assays and in animals with use of retroviral vectors to deliver antisense RNA. We still intend to use that strategy, but have first focused on cell-associated proteins rather than soluble ones. This strategy is based on the philosophy that soluble tPA may arrive in the tumor from the bloodstream, so that shutting down expression of tPA in endothelial cells may produce little effect. Therefore, we first obtained the murine cDNAs for MT1-MMP, annexin II and uPAR by long-distance RT-PCR from Swiss 3T3, EOMA, and mouse kidney total RNA using primers with Sal I restriction sites appended to the 5' ends. These cDNAs were ligated into a cloning vector (pGEM5zf(+)) and inserts in several recombinant plasmids for each cDNA were completely sequenced to identify a clone without PCR-induced errors. The plasmids with correct sequence were amplified in bacteria and digested with Sal I to liberate the cDNA. Following gel purification, cDNAs were ligated into pLNCX2 and recombinant plasmids with the inserts in the sense and antisense direction identified. This process was completed earliest for the MT1-MMP cDNA, so those plasmids were used in a test of the retroviral packaging/transduction system.

Phoenix-Eco cells[14] obtained from the ATCC through a technology transfer agreement with Dr. Garry Nolan of Stanford University were transfected with pLNCX2 without an insert, pLNCX2 with sense-oriented MT1-MMP, or pLNCX2 with antisense-oriented MT1-MMP. One-tenth concentration of a GFP-expressing plasmid, pLEGFP-N1, was included in the transfection to assess transfection efficiency (Figure 11).

Following transfection, virus was collected for 24 hours and the virus-containing medium applied to EOMA cells. G418 selection of the transduced EOMA cells revealed multiple colonies of G418-resistant cells in the transduced cultures, while cultures not exposed to virus were completely killed by G418 treatment.

To test the efficacy of the antisense vector to abrogate expression of MT1-MMP, the G418 resistant cells in EOMA cultures transduced with pLNCX2-containing virus or with virus containing pLNCX2 with MT1-MMP in the antisense direction were expanded in culture and used for immunofluorescence for MT1-MMP (Figure 12). Immunofluorescence was performed using a mouse monoclonal antibody for MT1-MMP that recognizes the mouse protein (IM57, Oncogene Research). Careful examination of four fields per chamber revealed reduced or absent MT1-MMP immunofluorescence in cells transduced with the antisense vector, while those transduced with the vector lacking inserted sequences expressed membrane-associated MT1-MMP (Figure 12). It would appear that this vector is ready to use in the mammary vessel assays.

KEY RESEARCH OUTCOMES

- Successful adaptation of the aortic ring assay to mammary fat pad vessels.
- Development of the ability to do immunofluorescence in fibrin matrix.
- Differential expression of MT1-MMP and tPA confirmed in existing cDNA with GAPDH control. Possible upregulation of uPA and uPAR identified.
- Construction of sense and antisense retroviral vectors for MT1-MMP, annexin II and uPAR.
- Transfection of packaging cells and production and titration of recombinant retrovirus.

REPORTABLE OUTCOMES

Watson, P.A., Hannum, R.S., Emanuels, A.E., and **McLeskey, S.W.** Differential gene expression in tumor-associated endothelium. 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002.

McLeskey, S.W., Watson, P., and Passaniti, A. Ex Vivo Angiogenesis Assay Using a Mammary Vessel Explant, 94th Annual Meeting of the American Association for Cancer Research, Washington, DC, July, 11-14, 2003

CONCLUSIONS

These studies have increasing importance because of the success of an antibody-based antiangiogenic therapy in patients with metastatic colon cancer[15]. The antibody in these studies was directed against VEGF-A, a very important angiogenic factor. However, previous to this trial, there were multiple trials of multiple antiangiogenic therapies in multiple types of cancer that produced disappointing results. The successful trial is proof that this approach may be a viable form of therapy in cancer, but it does not explain the failure of similar approaches in other trials. Therefore, although the current success can serve to spur us on, the challenge remains to identify viable antiangiogenic targets in breast and other cancers. Thus, the work we are doing with the mammary vessel assay has become even more important with the success this antiangiogenic trial.

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Appendices

1. List of acronyms.
2. Abstracts presented to the American Association for Cancer Research.
3. Figures 1-12

List of Acronyms

ACA	ε-amino-n-caproic acid
AcLDL	Acetylated low density lipid
AR	Aortic ring
ATCC	American Type Culture Collection
cDNA	copy DNA
DMEM	Dulbecco's minimal essential medium
DiI	A red fluorophore
EGF	Epidermal growth factor
EOMA	A mouse hemangioma cell line
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
LDL	Low density lipid
MFP	Mammary fat pad
MT1-MMP	Membrane type matrix metalloproteinase 1
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PBS	Phosphate buffered saline
PECAM	Platelet endothelial cell adhesion molecule
pLNCX2	A retroviral expression vector
PN-1	Protease nexin-1
PCR	Polymerase chain reaction
RNA	Ribosenucleic acid
RT-PCR	Reverse transcriptase – polymerase chain reaction
tPA	Tissue plasminogen activator
UMB	University of Maryland at Baltimore
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial cell growth factor

Abstract presented at the 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002

Differential Gene Expression in Tumor-Associated Endothelium

Watson, P.A., Hannum, R.S., Emanuels, A.E. and McLeskey, S.W.
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Baltimore, MD 21212

Although tumor-associated blood vessels have unique morphology and function, little is known about expression of genes that might determine their phenotype. We have performed a one-step flow cytometric separation of endothelial cells from MCF-7 xenograft tumors growing in nude mice or from mouse mammary fat pad. RNA was immediately extracted from the sorted cells and subjected to amplified fragment length polymorphism analysis. To date, we have about 30 candidate genes that may be differentially expressed and are confirming that expression in our xenografts and in human breast cancer. These genes include members of the tissue plasminogen activator/inhibitor family, proteins involved in cell-to-cell communication and motility, and unknown ESTs. Our current results concerning differentially expressed genes will be presented.

94th Annual Meeting of the American Association for Cancer Research, Washington, DC, July, 11-14, 2003

An *ex vivo* Angiogenesis Assay Using a Mammary Vessel Explant

McLeskey, S.W., Watson, P., and Passaniti, A.
University of Maryland Baltimore

Breast carcinomas recruit mammary gland vessels that enhance tumor growth and metastasis. To establish a more physiological model of breast cancer angiogenesis, we adapted the fibrin-based aortic ring assay to mammary vessels. In this assay, a segment of mammary blood vessel is placed in a fibrin matrix that stimulates the formation of tube-like structures composed of invasive endothelial cells. These vessels can be evaluated by immunofluorescence using antibodies reactive to molecules of interest. Young female mice were treated with subcutaneous estrogen pellets to stimulate mammary gland development. Two weeks following pellet implantation, the central vessel (the epigastric vein) of the inguinal mammary fat pad is harvested and cut into short segments. Chambered coverslips were prepared by thrombin polymerization of a small layer of a fibrinogen/growth medium mixture in the bottom of the chambers. Pieces of the mammary vessel were placed in chambered coverslips on top of the fibrin clot and another layer of fibrin was added to the top of the vessel pieces. After approximately one week, cells migrated from the explant into the matrix, some forming branched, tube-like structures. A subset of the tubular structures is composed of endothelial cells, which can be identified by immunofluorescence for PECAM-1, von Willebrand factor, and DiI-labeled acetylated LDL uptake. The chambered coverslip enables analysis by confocal microscopy, with 3-dimensional reconstruction of the endothelial network. Development of this *ex vivo* angiogenesis assay specific to breast endothelium in a matrix relevant to the carcinoma environment will enable elucidation of the role of fibrinolysis-associated molecules in breast tumor angiogenesis and will have applicability for other molecular families and matrix materials.

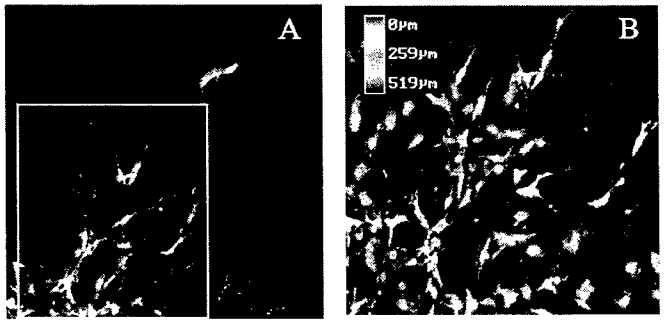

Figure 4. Confocal microscopy of mammary vessel assay. Fragments of mammary fat pad vessel were embedded in fibrin in one corner of a chamber slide (out-of-frame, lower left-hand corner) with a collagen pellet containing 10,000 tumor cells in the opposite corner (out-of-frame, upper right-hand corner). After two weeks, tubular structures have invaded the gel. **A.** Staining with Yo-Pro-1, a fluorophore that stains all cells. Branching structures are seen emanating from the explant. **B.** A 3-diminensional reconstruction of a 520 µm deep section of the same area of the gel (outlined by square in A), showing branching structures. Warmer colors are in higher focal planes.

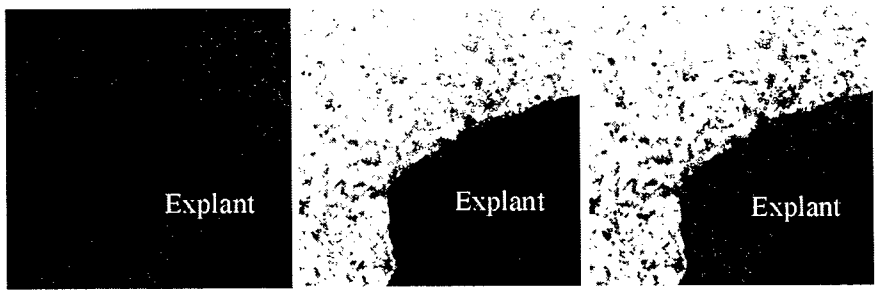

Figure 6. Live imaging of DiI-AcLDL uptake in mammary vessel implants growing in fibrin matrix. Mammary vessel explants were placed in fibrin matrix made with medium 199(E) supplemented with 10% fetal bovine serum and FGF (25 ng/ml). Multiple cells exit the explant, invade the fibrin, and are positive for DiI-AcLDL uptake (red cells in 2 panels on the left and in merged images). Some of the positive cells form linear structures.

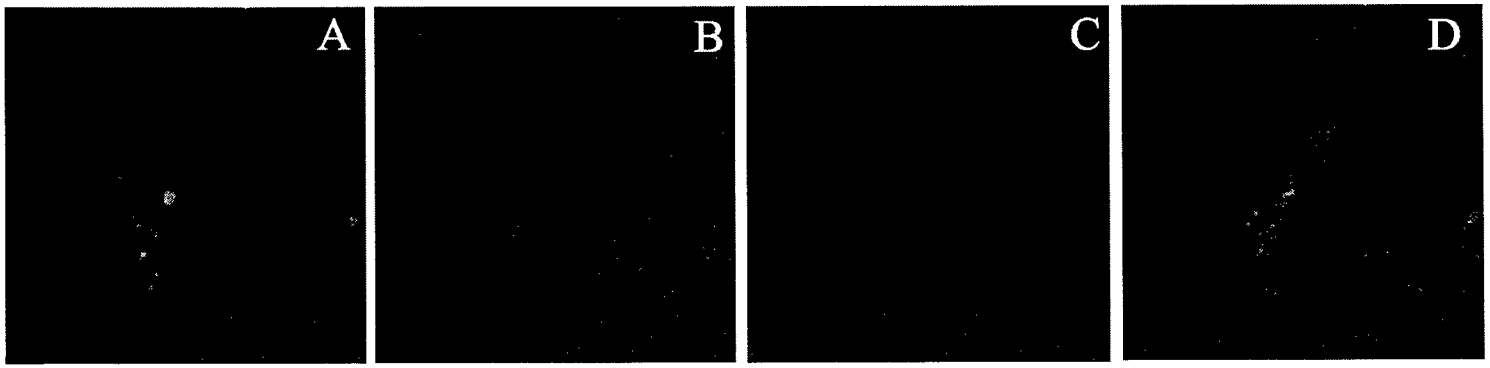

Figure 5. Confocal microscopy of mammary vessel explants in fibrin matrix. A-C. A branching structure is seen emanating from the mammary vessel explant. Green shows Yo-Pro-1 staining of nuclei, red is DiI-LDL uptake, and blue is cy-5 immunofluorescence for PECAM-1. **D** is a merged image of A-C. **Method:** Four days prior to harvest, DiI-labeled LDL (Biomedical Technologies, #BT902) is added to a concentration of 10 micrograms/ml. Fibrin cultures are fixed for 2 hours at 4° with 2% paraformaldehyde in PBS with 0.15% Triton X 100 followed by Dent's fixative (4 parts methanol and 1 part DMSO) overnight. Cultures are washed twice in absolute methanol for 20 minutes each followed by incubation for at least 1 hour in absolute methanol at -80°. After being brought to room temperature, cultures are incubated for 2 hrs in Dent's fixative plus 1 part H_2O_2, washed twice for 10 minutes each in PBS and primary rat anti-PECAM-1 antibody (PharMingin, #5533705, 5 micrograms/ml)is applied for 1 hour in 0.1% gelatin in PBS. Following three 5-minute washes with PBS, biotinylated antirat secondary antibody (Vector #4001, 5 micrograms/ml) is applied in 0.1% gelatin for 30 minutes. Following three 5-minute washes in PBS, cy-5 streptavidin (Jackson #016-170-084, 9 micrograms/ml) and Yo-Pro-1 (Molcular Probes, #Y3603, 1.3 micromolar) are applied in 0.1% gelatin in PBS. Following two 5-minute washes with PBS, the cultures are overlain with PBS for confocal microscopy.

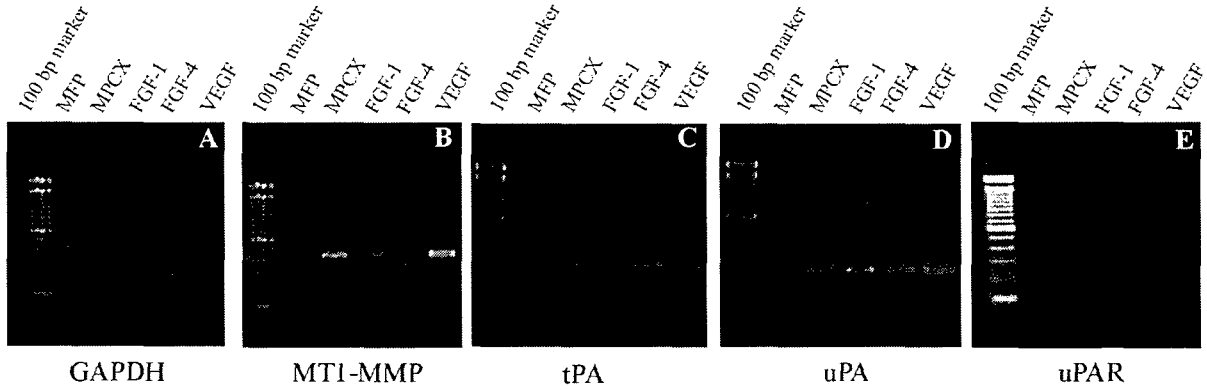

Figure 1. PCR of plasmid libraries standardized to GAPDH. PCR with specific primers for the indicated cDNA was performed on plasmid libraries isolated from tumor-associated endothelial cells. Amounts of each library used in all reactions were adjusted according to ratios of the individual libraries which gave the same intensity GAPDH band.

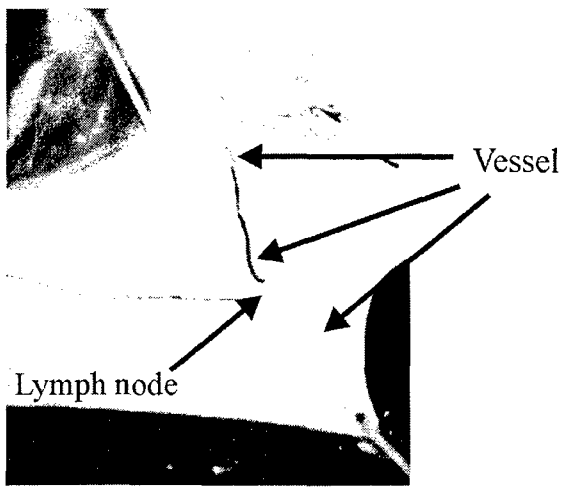

Figure 2. Mammary vessel dissected for mammary vessel assays. The right abdominal skin is reflected back, exposing the inguinal mammary fat pad. The vessel, which is probably the superficial epigastric vein, runs dorsally in the center of the inguinal mammary fat pad from the nipple area, at the bottom right of the picture. It intersects another vessel at a point where there is an inguinal lymph node. This node is somewhat enlarged in this animal, probably because it had a small traumatic lesion on its tail.

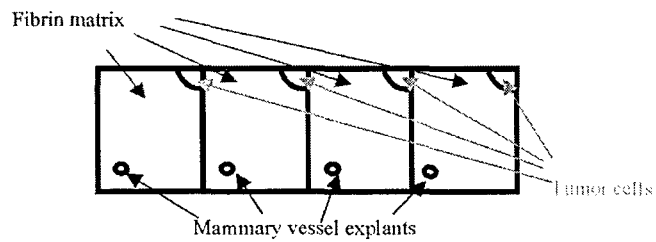

Figure 3. Cartoon depicting mammary vessel assay. Mammary vessel fragments are embedded in a fibrin or collagen gel. Endothelial sprouts invade the gel in the direction of the angiogenic stimulus (tumor cells). **Method:** Fibrinogen (3mg/ml) and amino caproic acid (0.3 mg/ml) are dissolved in DMEM 10% FBS and filtered through a 0.45 micron filter. Thrombin (100 units/ml) is added and 250 microliters placed in each chamber. Collagen pellets containing cancer cells, if desired, are positioned in the bottom layer. After the bottom layer is polymerized, the vessel fragment is positioned and 250 microliters additional fibrinogen is added and polymerized. Cultures are overlain with an additional 300 microliters of DMEM/10% FBS and incubated at 37° for approximately 8-14 days.

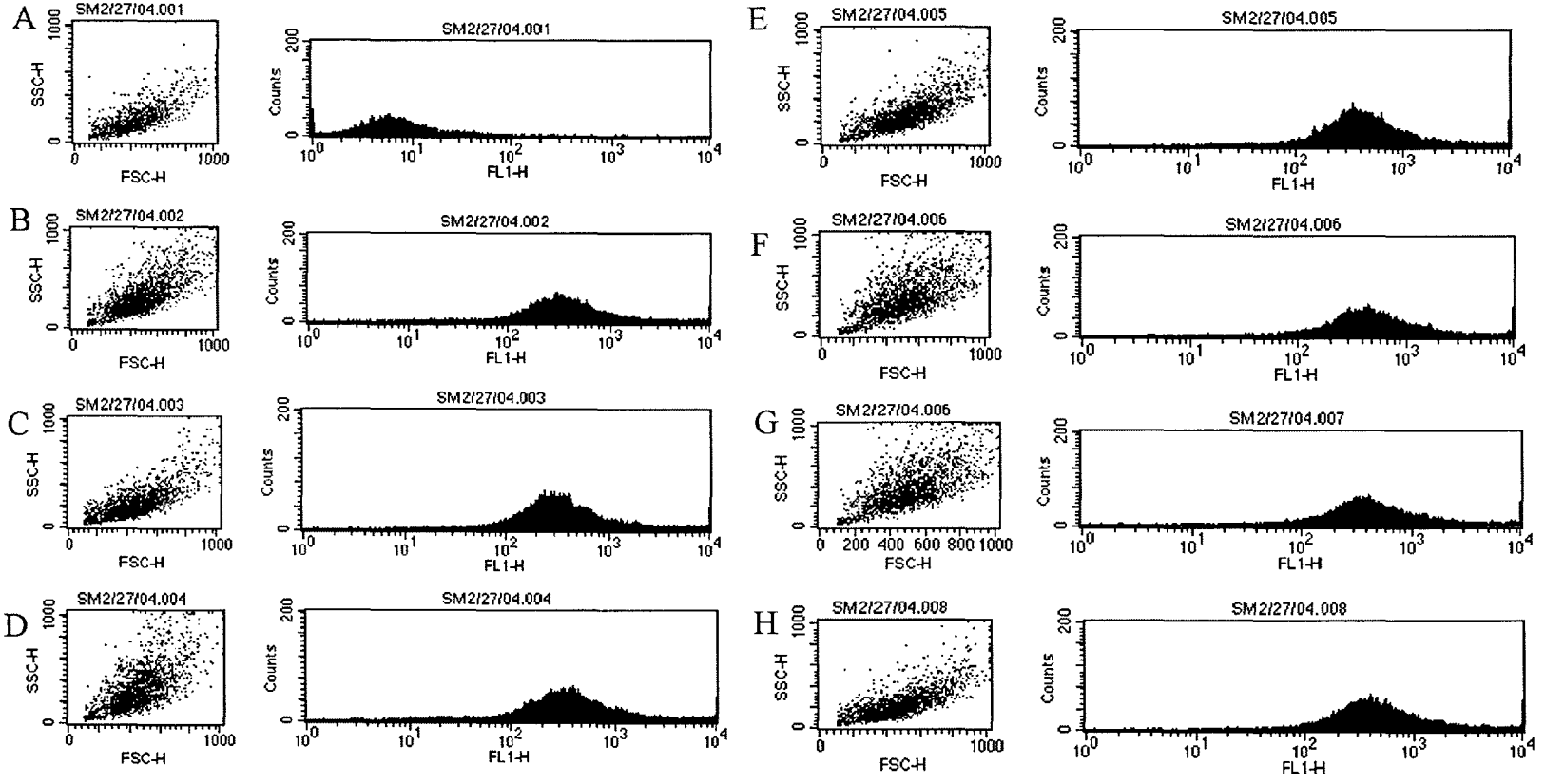

Figure 7. Flow cytometry analysis of HMEC-1 cells treated (B-H) or not treated (A) with AlexaFluor 488 labeled AcLDL. Treatments were as outlined in Table 1. **A** - Basal medium only. **B** - BPE. **C** - VEGF. **D** - FGF. **E** - BPE + VEGF. **F** - BPE + FGF. **G** - VEGF + FGF. **H** - All.

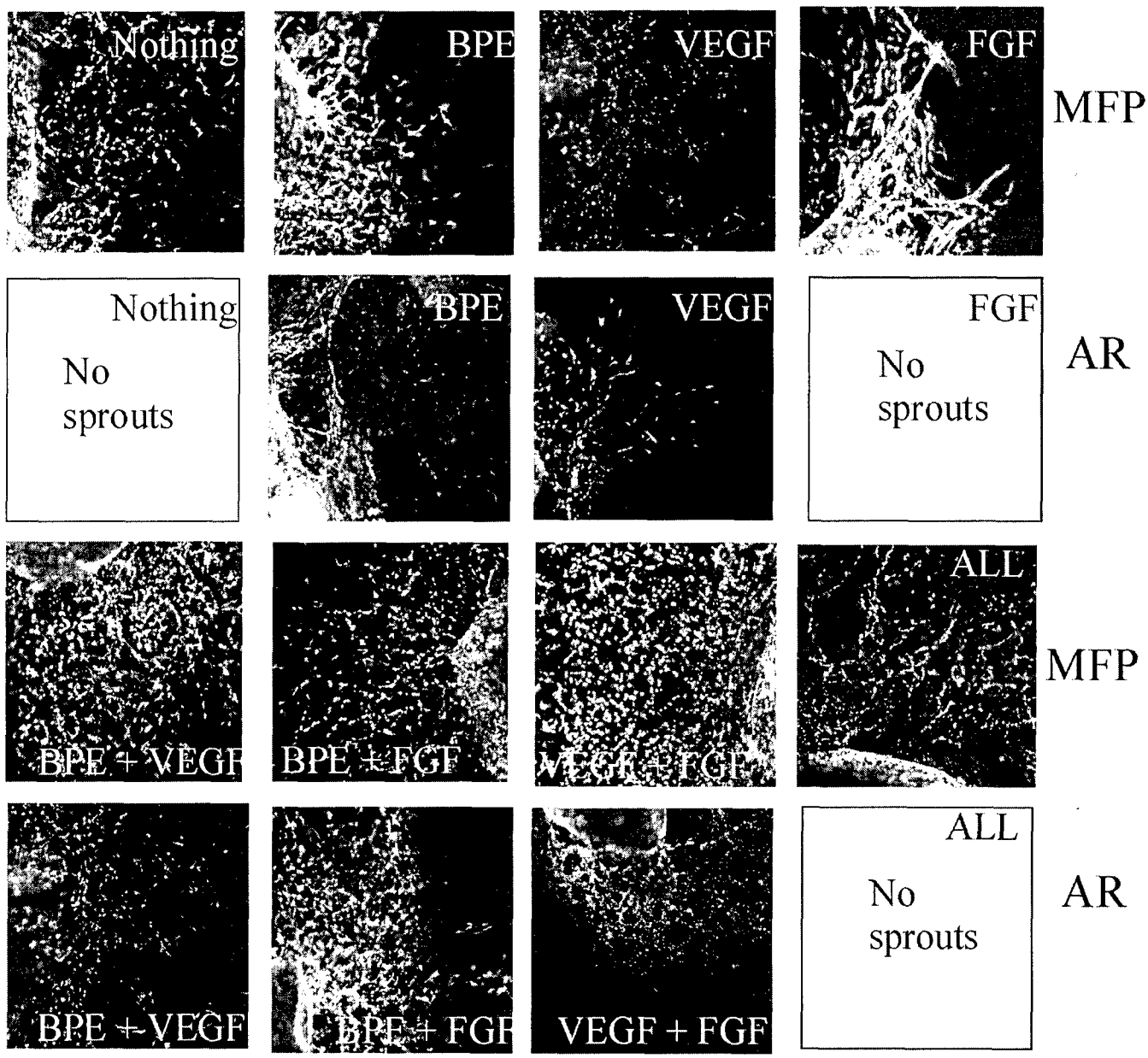

Figure 8. Mammary vessels (MFP) and aortic rings (AR) explants growing in fibrin matrix. Representative chambers of 4 per treatment are shown. Concentrations of treatments are given in Table 1. Green is Yo-Pro-1 staining. Yellow signifies that green and yellow staining are co-localizing. A square with "No Sprouts" indicates that no sprouts were observed leaving the explant under that treatment condition.

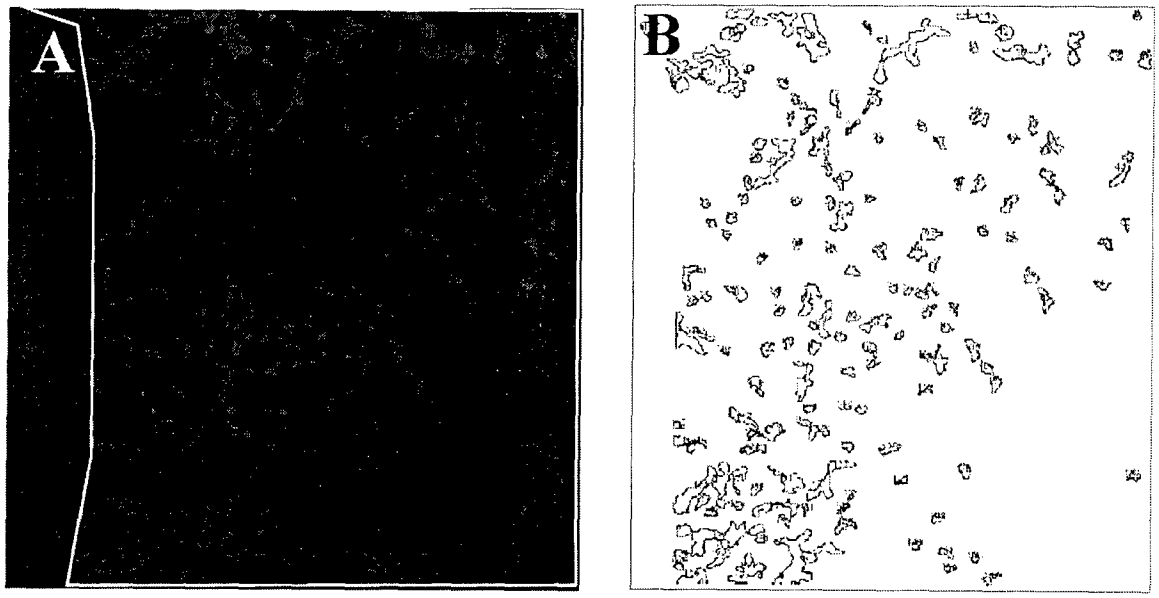

Figure 9. Example of image analysis of a mammary fat pad explant using ImageJ software. The thresholded image is shown in A, with the region of interest outlined in yellow. In B, areas of endothelial cell outgrowth identified by the program are outlined. A count of all distinct areas identified and the area of each are computed by the program.

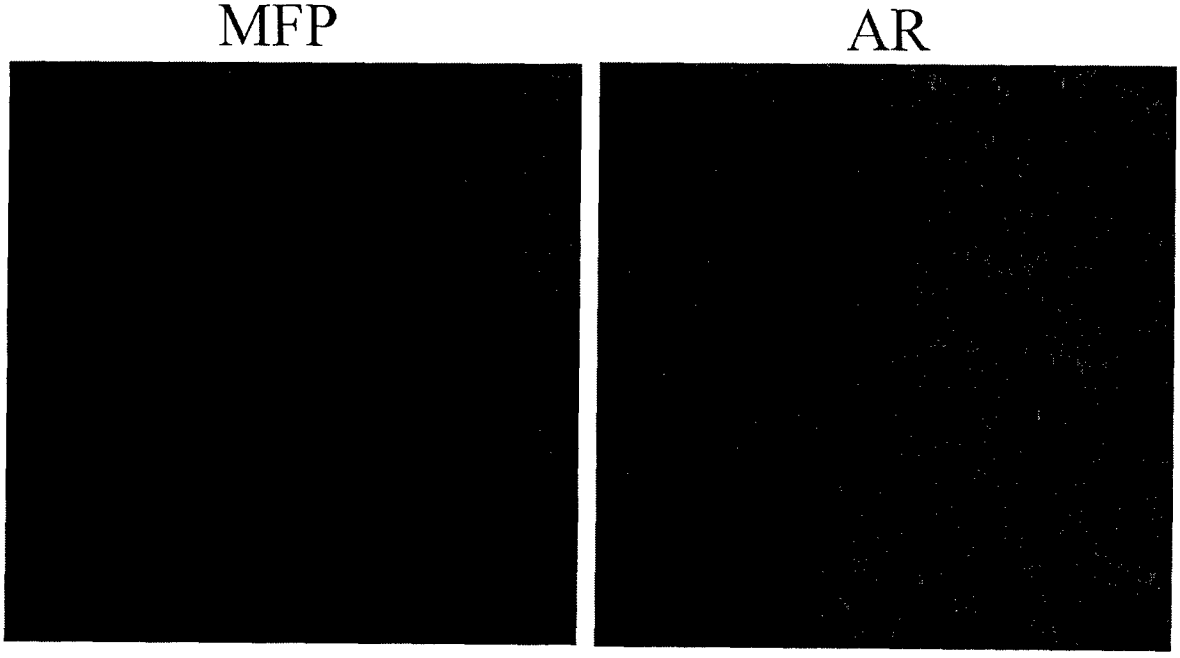

Figure 10. Three dimensional projection of tubular structures sprouting from a mammary vessel (left) or an aortic ring (right) growing in fibrin matrices. Red fluorescence is DiI-labeled Ac-LDL. This view is at approximately 70 degrees from the plane of each section.

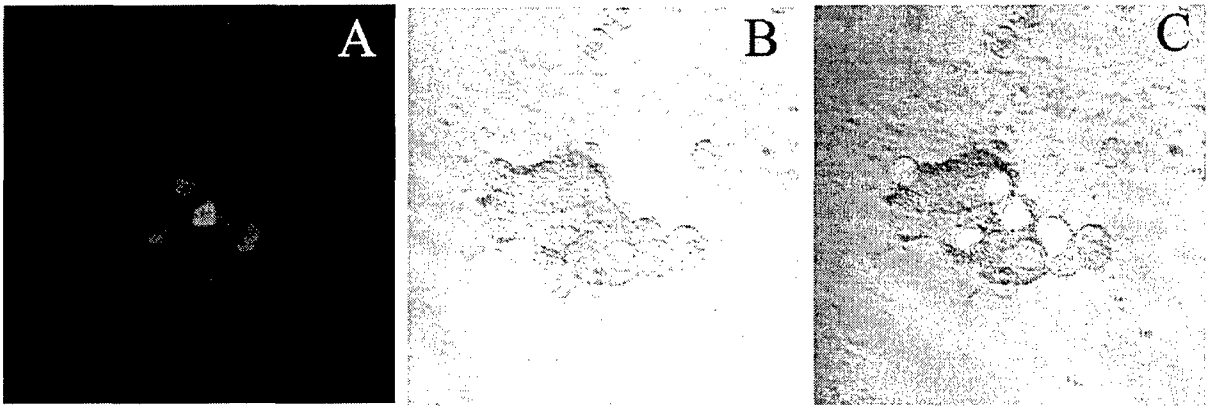

Figure 11. Transfected ecotropic packaging cells express green fluorescent protein. A. Green fluorescence is exhibited by about 7 packaging cells in a cluster. **B.** Nomarski image of the same field, showing about 30 cells in the cluster.. **C.** Overlay of the two images. **Method:** Phoenix-Eco cells were transfected with retroviral plasmids pLNCX2 or PLNCX2 containing inserts for sense-oriented or anti-sense oriented murine MT1-MMP. PLEGFP-N1, a retroviral plasmid directing expression of GFP was included at 1/10 the concentration to determine transfection efficiency. After harvest of viral supernatants, which were used to infect murine EOMA cells, transfected packaging cells were plated in a chambered coverslip for live fluorescence imaging.

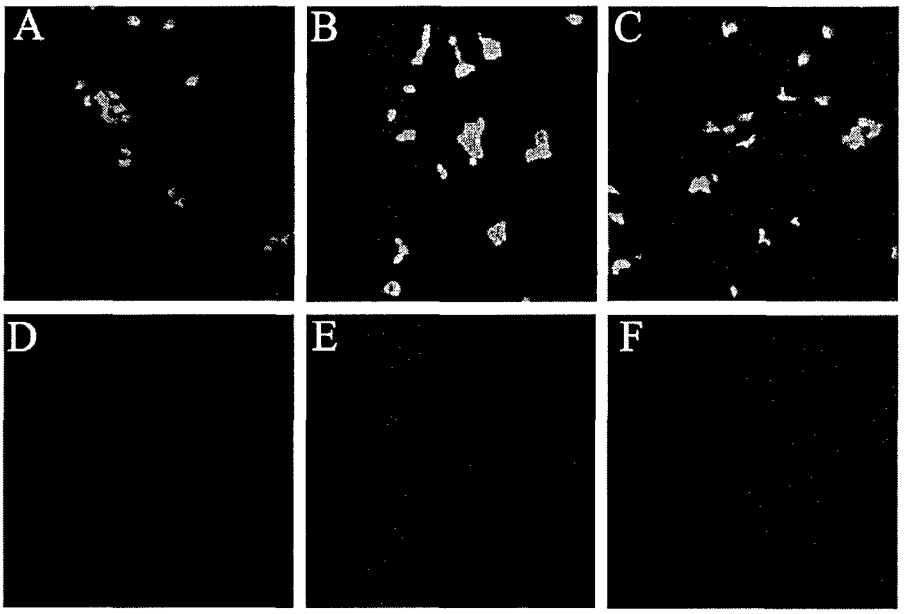

Figure 12. Transduction of EOMA cells with an antisense retroviral vector abro-gates expression of MT1-MMP. In panels A, B, and C, Yo-Pro-1 staining identifies all cells. In panels D, E, and F, immunofluorescence for MT1-MMP is observed only in cells transduced with an empty retroviral vector (E) and not in cells where the primary anti-MT1-MMP antibody was omitted (D) or in cells transduced with the MT1-MMP antisense retroviral vector (F). These are representative fields of 4 per chamber (200X). **Method:** Chambered coverslips were plated with transduced EOMA cells and stained with a mouse monoclonal anti-MT1-MMP using a biotinylated secondary antibody and cy5-strepavidin disclosure of MT1-MMP immunofluorescence.